DIFFERENTIAL DISPLAY OF DEHP AND TRENBOLONE INDUCED GENE TRANSCRIPT PATTERNS IN FATHEAD MINNOW LIVER

Reddy, TV*[1], Miracle, A [1],Flick, R [1], Lazorchak, JM [1], Smith, ME [2], Lattier, DL [1], and Toth, GP [1],
[1]U.S. EPA, Cincinnati, OH, [2]SoBran Inc., c/o U.S.EPA Cincinnati, OH

US EPA Office of Research and Development

Abstract:

The endocrine disruptor risk assessment process is being delayed without more robust data on the estrogenic and androgenic activity of chemicals found in the environment such as trenbolone and Di(2-ethylhexyl) phthalate (DEHP). Trenbolone is an androgenic compound known to reduce vitellogenin (Vtg) blood protein levels in female fathead minnows (*Pimephales promelas*). The plasticizer di (2-ethylhexyl) phthalate (DEHP) is known to be antiandrogenic and has been detected in fish tissue. In this study, we used '*fluoro* differential display' to investigate the global gene expression pattern in the livers of male fathead minnows exposed to trenbolone or DEHP. Adult (8-10 months old) male fish were continuously exposed to moderately hard reconstituted water (MHRW) as control, and MHRW with 10 µg/L of DEHP, for one week. Both male and female fathead minnows were exposed to *alpha*-trenbolone (175 to 7000 ng/L) or *beta*-trenbolone (50 or 500 ng/L) for 21 days. Total liver RNA was isolated, and reverse transcribed using an anchored oligo-dT primer. The cDNAs were subsequently amplified in a PCR reaction using fluorescent TMR-labeled anchored oligo-dT primers, in conjunction with four separate arbitrary decamers. The PCR products were separated on a 5.6% polyacrylamide gel, and visualized with a fluorescent scanner. Several bands were identified that exhibited differential expression among fathead minnows exposed to trenbolone, DEHP and controls. These bands were excised from the gel, and the cDNAs were eluted into buffer, re-amplified, and sequenced using an MJ Research BaseStation sequencer. Acquired nucleotide sequences were compared to sequences in GenBank using Blastn and Blastx programs to identify fathead minnow gene homologs.

Introduction:

Trenbolone is an androgenic steroid used extensively in implants used for cattle and horses to increase their musculature and body weight gains. As a result, trenbolone (alpha or beta) has been detected at confined animal feed operations (CAFO) effluent discharge sites and are of potential environmental concern. DEHP is plasticiser used in the plastic industry. DEHP is an ubiquitous environmental contaminant and has been detected in fish tissue and in human blood samples. DEHP has been shown to have antiandrogenic effect in male rats.

Differential display (Liang and Pardee, 1992) is a technique useful in the identification of genes that are differentially regulated. Initially, mRNA is reverse transcribed into cDNA using 'anchored' primers that anneal to the poly (A+) tail. From the resulting pool of cDNAs, random gene fragments are amplified using anchored primers together with random 'arbitrary' primers. When these fragments are electrophoresed, patterns of expression can be compared by noting the bands that at are present or absent between the treatment groups.

We used the technique of differential display to screen for potentially differentially expressed genes in fathead minnow (*Pimephales promelas*) exposed to DEHP for 7 days or *alpha*- and *beta*-trenbolone for 21 days. Our goals for this work were 1) to assess the potential for application of differential display in fathead minnow and 2) to determine if fathead minnow exhibits any differential gene expression when exposed to trenbolone or DEHP and to identify these genes, and finally validate these genes by using semi-quantitative real time-PCR (QRT-PCR).

Materials and Methods:

Test Species: Fathead Minnow (*Pimephales promelas*)

Fathead minnow adult fish were held under standard conditions following established in-house SOP's. The test was initiated using 30 L of control moderately hard reconstituted water (MHRW) or test treatment water (MHRW mixed with 10 µg/L DEHP) per tank. Twelve adult male fathead minnows were maintained in each control or treatment tank. As fish were removed for necrospy (5 or 7 fish / group), at the designated interval (4 or 7 days post exposure), the volume of test solution was reduced accordingly, so that the loading rate of 2 L of test solution/fish exposed remained constant. Fish were fed 0.1 g tropical fish flake food daily.

Water was renewed in all control or test treatments daily by removing 80% of the old water and replacing it with freshly prepared control test solution. Accordingly, freshly made dosing solutions (solvent control DMSO or DEHP) were added daily to the control and experimental tank.

Test Chemicals: Di(2-ethylhexyl) phthalate (DEHP), *alpha*- or *beta*- trenbolone.
Chemical Structures:
Treatment:

DEHP: Fathead minnows where exposed daily to DEHP (10 µg/L) and sacrificed at the end of 4 or 7days. Livers were isolated and stored in RNA Later® solution (Ambion, Inc.), in accordance with manufacturer's specifications for isolation of RNA.

Trenbolone: EPA, ORD, NHERL, Mid-Continent Ecology Laboratory-Duluth exposed Liver, ovaries and teste tissues from fathead minnows, to alpha trenbolone (175 to 7000 ng/L), or beta trenbolone (50 or 500 ng/L) for 21 days. Tissues were placed in RNA Later from for further analyses.

Methods:

Total RNA from tissues (liver,ovaries and testes) was isolated by the standard guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987).

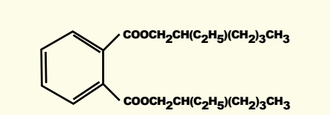
Differential display was performed using the Hieroglyph mRNA Profile System with the fluoroDD fluorescence kit (Beckman Coulter).

Total RNA was quantified after isolation by UV spectrophotometry. 0.2 µg of total RNA was reverse transcribed into cDNA using Superciript II reverse transcriptase (Gibco-BRL, Inc.), and anchored oligo-dT primers. The resulting cDNAs were subsequently amplified in a PCR reaction using fluorescent TMR-labeled anchored oligo-dT primers and one of several arbitrary 10 base pair oligonucleotides. PCR products were separated on a 5.6% polyacrylamide gel and visualized with a fluorescent scanner. Bands that appeared to exhibit differential expression, between fish exposed to DEHP/trenbolone and controls, were excised from the gel. Isolated gel slices were placed in 50 µl [10:1 Tris-EDTA] buffer to elute cDNA.

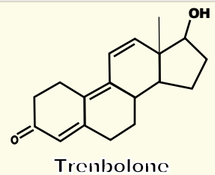
Aliquots (4 µl) of isolated cDNAs were re-amplified using M13 reverse primers and T7 promoter primers. PCR products were electrophoresed on a 1.2% agarose gel stained with ethidium bromide. Bands representing re-amplified products were carefully excised from the gel and purified using a QiaQuick spin column (Qiagen, Inc.), and processed for sequencing.

Sequencing :

Cycle sequencing reactions were prepared using DYEnamic ET Terminator sequencing reagents (Amersham, Inc.). Samples were subjected to electrophoreses on an MJ BaseStation sequencing apparatus and analyzed using Cartographer v.1.2.4 software (MJ Geneworks, Sauk City, WI). Corrected sequences were analyzed against GenBank databases using blastn and blastx functions (NCBI, NHLBI, NIH) to determine potential sequence homology.



DI (2-ETHYLHEXYL) PHTHALATE



Trenbolone

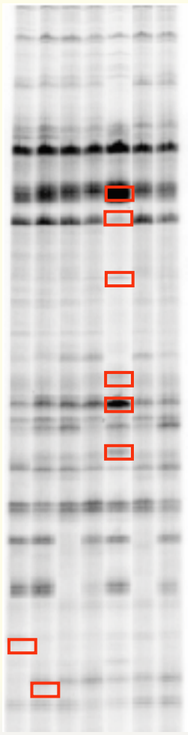


Fig: 1
Differently expressed Gene Transcripts in Trenbolone treated Fathead Minnow Liver Acrylamide Gel Electrophoresis

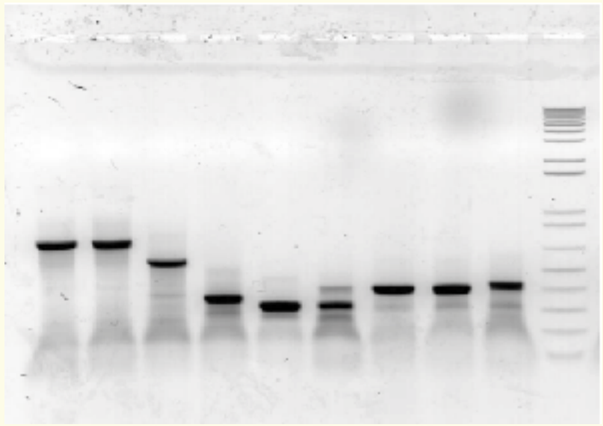


Fig: 2
Amplified Acrylamide bands followed by Agarose Gel Electrophoresis of differently expressed genes in liver exposed to 10 µg/L DEHP for 7 days.

QRT-PCR:

In order to validate the magnitude of differential gene expression for the genes isolated by differential display, semi-quantitative reverse transcription-PCR (QRT-PCR) was performed for selected genes. 0.2 µg of total RNA, for each gene of interest, was reverse transcribed to synthesize single strand cDNA by mixing RNA with 10 mM dNTP, 25 mM $MgCl_2$, 2 µl 10 X buffer, 1 µl RNase inhibitor, 1 µl random hexamers, and 1 µl MuLV reverse transcriptase (all reagents Applied Biosystems). A no amplification control was created to check for the presence of contaminating genomic DNA by omitting the addition of RT enzyme to one of the DEHP-exposed RNA samples. Reactions were heated to 48ºC for 30 minutes followed by 95ºC for 5 minutes to inactivate the RT. Samples were diluted to a final volume of 100 µl with de-ionized water.

Standards of calculated mass, for the various genes, were amplified from differential display fragments generated as described above. Primer sequences for standards and QPCR analyses were designed using OLIGO 6.58 software (Wojciech and Rychlik). Standards were prepared in 10-fold serial dilutions, in order to generate mass-specific curves for comparison of sample amplifications

'Real time' polymerase chain reactions (QRT-PCR) were performed on the MJ Opticon (MJ Research) using 3 µl of cDNA -*or*- 2-4 pmol of respective standards. In addition to optimized concentration of oligonucleotide PRC primers, 12.5 µl 2X SYBR Green master mix (Applied Biosystems), and 7.5 µl water were also added. No template controls were created for each primer set by omitting the addition of cDNA or standard template to the reaction. Cycling parameters included an initial denaturation step of 10 minutes at 95ºC, in order to activate the T*aq* polymerase. The PCR profile contained the following cycling parameters: 40 cycles of 15 seconds at 95ºC and a 1 minute annealing step at 60ºC were performed, with a CCD scan taking place following each complete cycle to detect double-stranded DNA product. A melt curve analysis was performed at the end of PCR cycling with CCD scans [-dT/dF] of each sample well occurring every 10 seconds at one degree increments in temperature - from 55ºC to 95ºC. Samples were given cycle threshold values based on product formation compared to the standard curve generated for each gene.

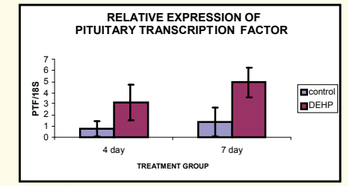


Fig: 3
4 day exposure represents a 4-fold increase in relative PTF expression (N=5), 7 day exposure represents a 3.5-fold increase in relative PTF expression (N=4 or 7) ± Standard Deviation

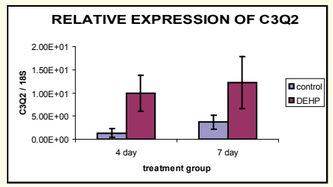


Fig: 4
4 day exposure represents a 7-fold increase in relative C3Q2 expression (N=5), 7 day exposure represents a 4-fold increase in relative C3Q2 expression (N=4 or 7) ± Standard Deviation

Results & Discussion:

- Fish exposed to trenbolone and DEHP shows differentially expressed gene patterns on acrylamide gel (Fig. 1), and were purified for sequencing by reamplification (Fig. 2).
- Analysis of the trenbolone-exposure generated gene sequences identified genes homologous to zebrafish fatty acid binding protein (testes), carp ATP synthase B subunit (liver and testes), and human calumenin (testes).
- Similarly, fathead minnows exposed to DEHP showed differentially expressed genes in liver that are homologous to an apolipoprotein variant similar to a 14 kDa variant known in the eel, *Anguilla japonica;* rat diamine oxidase, carp pituitary-specific transcription factor, carp complement C3H1, C3H2, C3Q2, murine inter-alpha trypsin inhibitor heavy chain type 2, and human desmoglobin.
- Further validation of pituitary transcription factor expressed in fathead minnow liver exposed to DEHP (10 µg/L) for 4 and 7 days, showed a 4- and 3.5-fold increase, respectively, over controls (Fig. 3).
- Complement gene C3Q2 however, showed a 7-fold increase in levels of mRNA in the liver following exposure to DEHP for 4 days and a 4-fold increase after 7 days of exposure to 10 µg/L with respect to controls (Fig 4).

Conclusions:

The differential display technique and QRT-PCR are powerful techniques that can be employed to discover novel genes expressed in organisms for which there is limited genomic information. In turn, the genes identified in differential gene analyses may be used as tools in exposure monitoring.

References:

Chomczynski, P. and Sacchi N. 1987. Single step method of RNA isolation acid guanidinium thiocyanate-phenal-chloroform extraction. Anal Biochem 162: 156-159.
Liamg, P. and Pardee, A.B., 1992, Differential display of eukaryotic mRNA by means of the polymerase chain reaction. Science 257: 967-971

Disclaimer:

The views expressed in this poster are those of the author's and do not necessarily reflect the views or policies of the US EPA nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Prepared by CSC, Inc. for ORD/NERL/Cincinnati, OH - November 2002